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Sir:

Applicants hereby claim priority under 35 U.S.C. §119 from European patent application no. 00204207.5, which was filed on November 27, 2000. A certified copy of the European application from which priority is claimed is enclosed.

Respectfully submitted,

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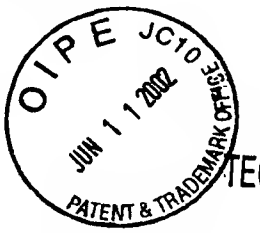

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The attached documents are exact copies of the European patent application described on the following page, as originally filed.

Les documents fixés à cette attestation sont conformes à la version initialement déposée de la demande de brevet européen spécifiée à la page suivante.

Patentanmeldung Nr. | Patent application No. | Demande de brevet n°

00204207.5

Der Präsident des Europäischen Patentamts;
Im Auftrag

For the President of the European Patent Office

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Blatt 2 der Bescheinigung
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Page 2 de l'attestation

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(42)

PROCESS FOR FOLDING POLYPEPTIDES AND PROTEINS PRODUCED BY CHEMICAL SYNTHESIS

FIELD OF THE INVENTION

The present invention relates to a process for folding chemically synthesized polypeptide and protein derivatives.

BACKGROUND ART

Over the last decade or so there has been an escalation in the demand of synthetic proteins after the successful chemical synthesis of fully active HIV-protease, a 99-residue enzyme prepared by highly optimized methods of solid-phase peptide synthesis (SPPS) based on the standard Boc/Bzl approach.

The synthesis in 1994 of crystalline ubiquitin, a small protein consisting of 76-residues, has further demonstrated that highly pure proteins can be synthesized by SPPS based on the Fmoc/t-Bu protocol, a method operationally simpler and chemically less complex than the Boc/Bzl procedure.

As of 2000, there is ample experimental evidence that single domain proteins containing between 60 and 100 amino acid residues can be produced rapidly, reliably and economically by chemical synthesis, with the assistance of a peptide synthesizer, in amounts that are sufficient for structural and functional studies (P. Lloyd-Williams, F. Albericio, E. Giralt, " Chemical Approaches to the Synthesis of Peptides and Proteins ", CRC Press LLC : Boca Raton, FL, 1997).

More or less successful syntheses of proteins containing disulfide bonds are continuously reported but further improvements of the existing chemistry and folding strategies are needed to solve still persisting problems in the practical preparation of native cystine proteins and their analogues.

Proteins containing disulfide bridges prepared by chemical synthesis, once folded, have the same

properties as natural and genetically engineered forms. Disulfide bridges of proteins form single or multiple intra- and/or interchain cyclic structures which impart considerable conformational restraints to the molecules thus decisively contributing to the stabilization of the bioactive conformation.

Single domain proteins of known structure can be unequivocally prepared by regioselective pairings of the cysteine residues (L. Moroder et al. Biopolymers (Peptide Science). Various combinations of cysteine protecting groups compatible with the commonly used protection schemes were developed that allow for stepwise and pairwise deprotection and/or cooxidation of cysteine residues with complete selectivity.

A demonstration of how demanding is the chemistry involved for regioselective pairing of the cysteine residues in proteins containing multiple cystine residues is the recent synthesis of an insulin-like peptide, human relaxin. The synthesis of the A chain precursor was carried out using SPPS methods, the Fmoc/t-Bu approach and a p-alkoxybenzyl alcohol-based resin, while the B chain precursor was prepared using PAM resin (4-carboxyamidomethylbenzyl ester linkage to polystyrene-based resin) and following the Boc/Bzl approach. Of the four cysteine residues of the A chain precursor, two were protected as the S-Trt (S-triphenylmethyl) derivatives, while the others had S-Acm (S-acetamidomethyl) and S-Meb (S-p-methylbenzyl) protection, respectively. The two cysteines of the B chain precursor were protected by S-Acm and S-Meb protecting groups. The intramolecular S-S bridge of A chain was obtained first by iodine oxidation in AcOH. Then, the two intermolecular disulfide bridges connecting A and B chains were obtained in two steps: in the first step, the free thiol of chain A precursor obtained by HF deblocking of the S-Meb protecting group was reacted with the activated Cys(Npys) (S-3-nitro-2-pyridine sulphenyl) residue of B chain (directed intermolecular heterodisulfide formation) and,

in the second step, the remaining S-S bridge was obtained by cooxidative removal of the S-Acm groups with iodine.

The laborious methods of regioselective cysteine bridging are in most cases successfully replaced by simpler procedures that guarantee high degrees of selectivity of the cysteine pairing process during folding of the polypeptide chain (T. E. Creighton "Protein Structure : A Practical Approach", IRL Press, Oxford, 199, 1989).

SPPS protocols offer the possibility to prepare by chemical synthesis a variety of protein and polypeptide derivatives containing the cysteine residues protected with the same blocking group. Once the protecting groups are removed by a number of oxidizing reagents the disulfide bonds form directly. Derivatives containing S-Trt or S-Acm protected cysteines can be indeed efficiently folded upon treatment with iodine, N-iodosuccinimide and cyanogen iodide under carefully controlled conditions of solvent, pH and reaction time that will minimize modification of oxidation sensitive Tyr, Met, Trp and avoid overoxidation of cysteine thiols to the corresponding sulfonic acids.

Thallium(III) trifluoroacetate can replace the above oxidizing agents giving sometimes better yields of disulfide bonds. The major limitations of this reagent are its toxicity, the difficulty to remove thallium from the target polypeptide and the need to protect Met and Trp residues from oxidation.

Oxidizing reagents containing a mixture of sulfoxide/silyl compounds and trifluoroacetic acid have been successfully applied for the direct oxidation to disulfide bonds of polypeptide precursors containing S-Acm, S-But, S-Meb and S-Mob (S-p-methoxybenzyl) cysteine residues. The need to protect the Trp indole ring with formyl to avoid chlorination under oxidizing conditions is the major limitation of this mixture. This method has been also applied for direct disulfide formation on resin

with final yields higher to other reported yields in similar cases.

Methods for the oxidative folding of linear, synthetic polythiol precursors (reduced polypeptide forms) are, however, more popular and most frequently applied (S. Kubo et al., in Peptide Chemistry 1994, M. Onho, Ed., Protein Research Foundation, 313, 1995 ; S. Sakakibara, Biopolymers (Peptide Science), 37, 17, 1995). In the simplest method, the appropriate disulfide bonds can be spontaneously formed in the presence of air or some other mild oxidizing agents. Furthermore, folding and cysteine pairing is obtained in the presence of both the reduced (RSH) and oxidized (R-S-S-R) forms of a low molecular weight sulphydryl compound.

In synthetic polypeptides and single domain small proteins, the thermodynamic driving force for folding which results from a combination of H-bonding, ion pairing and hydrophobic effects is apparently substantial enough to spontaneously produce the native isomers in random renaturating oxidation (S. Kubo et al., Biopolymers, 38, 733, 1996). From studies of the oxidative folding of multiple cysteine-containing small proteins like enzyme inhibitors, toxins or hormones, useful informations have been also derived about particular structural motifs e.g. cysteine-stabilized beta turn (F. Siedler et al., Biopolymers, 34,1563, 1994), cysteine-stabilized poly(Pro)-II helix fold (E. Wunsch et al. Int. J.Peptide Protein Res., 37,61, 1991) and cysteine-stabilized alfa-beta-structural fold (B. M. P. Huyghues-Despointes and J.W. Nelson, Biochemistry, 31, 1476, 1992, K. Ramalingam and G.H. Snyder, Biochemistry, 32, 11155, 1993) whose stabilization is the main driving force for the correct disulfide bridging even in relatively small peptide molecules. If attention is paid to the choice of buffers, temperature and additives that will stabilize the secondary structural motifs, then even complete correct folding of partially folded or scrambled proteins can be obtained in vitro.

A number of folding protocols for polythiol species have been designed to :

a. minimize incorrect intramolecular cysteine pairing that leads to non-native, misfolded isomers ; and

b. avoid as much as possible random intermolecular disulfide bond formation that promote aggregation and precipitation.

Air oxidation is generally carried out at high dilution of the precursor polythiol form (c = 1 mg/ml or below) under neutral or slightly alkaline conditions (C.H. Li et al., Proc. Natl. Acad. Sci. USA, 80, 2216, 1980). It usually requires a long duration and produces an harmless by-product as water in the reaction.

Air oxidations are difficult to control because trace amounts of metal ions strongly influence their rates. More important, basic and hydrophobic precursor molecules tend to aggregate and precipitate out of the solution at or near their basic or neutral isoelectric points during the folding process. Furthermore, side-products due to oxidation of Met accumulate during folding. Although the number of chemical operations necessary to fold polythiol precursors is reduced to a minimum, the disulfide bridges formation promoted by the molecular oxygen of air gives in many instances low yields, sometimes not occurring at all.

DMSO and potassium ferricyanide have been also used as oxidants(J. P. Tam et al. J. Am. Chem. Soc. 113, 6657, 1991). Potassium ferricyanide must be used in the dark and, if Met and Trp are present in the polypeptide chain, oxidation side-products accumulate during folding. The use of DMSO often gives better results due to the fact that oxidative foldings can be conducted under acidic conditions at an efficient rate with no harmful products in the reaction. The method is particularly suitable for folding basic and hydrophobic polypeptide precursors due to the higher solubility characteristics of species undergoing oxidation in acidic buffers.

Problems in removing DMSO from the final product and reduction on the selectivity of disulfide bridges formation have been frequently reported. Furthermore, scrambling of disulfide bridges leading to misfolded isomers and oligomerization cannot be always avoided even with a careful control of the experimental conditions.

Higher yields of correct cysteine pairing and folding in small protein polythiol precursors are most frequently obtained by the use of redox buffers such as oxidized (GSSG) and reduced (GSH) glutathione and cystine/cysteine (Cys_2/Cys). During the oxidative foldings of Ribonuclease A (R.R. Hantgan et al. Biochemistry 13, 613, 1974), the 49 amino acid core domain of Hirudin (B. Chatrenet and J. -Y. Chang J. Biol. Chem. 267, 3038, 1992) and Bovine Pancreatic Trypsin Inhibitor (BPTI) (T. E. Creighton Methods Enzymol. 131, 83, 1986) induced by GSSG/GSH or Cys_2/Cys, free sulphydryls and disulfide groups are formed and reformed constantly throughout the folding process.

Overall rates and yields are usually better than oxidative folding in air because thiol/disulfide exchange occurring through thiolate intermediates facilitates the reshuffling of non-native disulfide to the natural ones. As for oxidative folding in air, high dilution of the polythiol precursor is necessary to avoid aggregation, formation of oligomers and polymers and to maximize yields of the target species.

As shown in detail, during the first stage of folding Hirudin $^{1-49}$ in vitro, folding proceeds sequentially and irreversibly from the unfolded, reduced form (polythiol) to equilibrated isomers containing one and two disulfide bridges and to equilibrated species containing three disulfide bonds (scrambled isomers) (J. -Y. Chang Biochem. J. 300, 643, 1994).

Nearly all 75 possible species, including the native one, have been identified : 15 isomers with one S-S bridge, 45 isomers with two S-S bridges and 15 isomers

with three S-S bridges. During the second stage of folding, the scrambled species reorganize by reshuffling the non-native disulfides to attain the native species. Disulfide formation is promoted primarily by oxidized glutathione or cystine, whereas disulfide reshuffling requires a thiol catalyst e.g. reduced glutathione or cysteine or mercaptoethanol.

The effectiveness of thiol reagents in promoting reshuffling was apparently related to their redox potential and each catalyst displayed an optimum concentration. Cystine/cysteine is about 10 fold more potent than GSSG/GSH in the process of accumulation of scrambled Hirudins. This difference has been explained by the relative redox potential of the GSSG/GSH (-0.24 V) and Cys-Cys/Cys (-0.22 V)systems (J. Rost and S. Rapoport Nature (London(201, 185, 1964 ; P.C. Jocelyn Eur. J. Biochem. 2, 327, 1967).

By selecting a combination of optimal conditions (temperature, buffer, salts and redox mixture) the process of folding of Hirudin $^{1-49}$ was accelerated to the extent that it reached completion within 15 min.

In general, the native conformation of a synthetic polypeptide or protein containing several disulfide bonds should form spontaneously under optimum conditions of folding polythiol forms. In many instances, however, even in optimized conditions oxidative foldings mediated by the above redox buffers do produce a considerable amount of by-products and mismatched forms cannot be avoided. This is the case of proteins that tend to form the native conformation only on the surface of specific membranes or under assistance of a specific molecular chaperon (S. Sakakibara Biopolymers, Peptide Science 51, 279, 1999).

Furthermore, despite their widespread use, most of the oxidative foldings of polythiol precursors promoted by air or the GSSG/GSH and Cystine/cysteine redox pairs have been conducted in a manner of trial and error, as clearly demonstrated by folding experiments of

synthetic chemokine and chemokine analogues (I. Clark-Lewis et al Methods Enzymol. 287, 233, 1997). In fact, while native chemokines and a number of their analogues fold readily, the folded structure being stabilized by two or three disulfide bridges, several analogues do not fold well under the same conditions as the corresponding native molecules and partially folded forms results. These observations represent a strong indication that changes in the primary structure of the polythiol precursors may adversely effect the induction of correct local folds (beta-turns, polyproline helical motifs etc.) in the polypeptide chains to be folded. Hence, the propensity to fold of many thiol precursors is mainly an intrinsic property of the polypeptide chain rather than a function of the specific oxidation system acting on the molecules.

Enhancement of selected disulfide pairings by adding alcohols, acetonitrile and DMSO to buffers at low ionic strength has been also reported (S. Snyder J. Biol. Chem. 259, 7468, 1984). The strategy involves enhancing formation of specific disulfide bonds by adjusting electrostatic factors in the medium to favor the juxtaposition of oppositely charged amino acids that border the selected cysteine residues.

Enzymes such as peptidyl disulfide isomerase (PDI) and prolyl isomerase (PPI) have been also employed as additives to catalyze and modulate disulfide exchange. The time required for folding Hirudin in vitro can be shortened from 10 h to 30 sec if PDI is added to the refolding buffer (J. -Y. Chang Biochem. J. 300, 643, 1994). In this case, the efficiency of folding in vitro does not differ significantly from that observed in vivo.

Polythiol polypeptide precursors are directly obtained by polypeptide-resin acidolytic cleavage when the cysteine residues are protected by acid-labile groups e.g. Trt. Alternatively and preferably, derivatives in which all cysteines are protected by an acid-resistant group e.g. the acetamidomethyl group (Acm) are first

isolated as S-cysteine derivatives by acidolytic peptide-resin cleavage and then the Acm group is eliminated by treatment with $Hg(AcO)_2$ in acetic acid, followed by the removal of Hg ions by gel filtration in the presence of a large excess of mercaptoethanol (R. A. Boykins Cytokine 11, 8, 1999).

In both cases, several side-reactions have been reported to occur at cysteine and tryptophan residues. The indole ring of tryptophan is can be derivatized by mercaptoethanol and cysteine gives a number of side-reactions, the most important being oxidation and alkylation by t-butyl cations during the acidolytic removal of the polypeptide chain from the resin.

Because of the shortcomings of the existing methodologies, there is a need for additional efficient and simpler procedures for folding polypeptide and protein precursors obtained by chemical synthesis.

Accordingly, it is an object of the present invention to provide an efficient folding method for synthetic polypeptide and protein derivatives containing protected cysteine residues.

It is another object to provide a folding method that minimize the formation of isomers containing mismatched disulfide bridges.

It is a further object to provide a folding method that does not utilize expensive disulfide-reshuffling reagents such as glutathione or enzymes. It still a further object to provide folding conditions that are simple, repeatable, robust and scalable. These and other objects will be apparent to those of ordinary skill in the art.

OBJECT OF THE INVENTION

It is the object of the present invention to provide a simple, rapid and robust method for the production of biologically active polypeptides and proteins containing disulfide bridges, from specific derivatives obtained by chemical synthesis.

SUMMARY OF THE INVENTION

The objective is achieved by the combination of classical stepwise solid-phase assembly of S-t-butyl-thio cysteine polypeptide derivatives and the finding that considerable amounts of native, biologically active forms can be produced by subjecting said derivatives to fold in the presence of cysteine at pH slightly above neutrality, at 37°C. Surprisingly, the production of biologically active polypeptides and proteins is achieved with a high molar excess of cysteine in a one-step procedure which is simpler and more efficient than the procedures described in the prior art for the folding of cysteine containing polypeptides and proteins obtained by chemical synthesis.

More specifically, the invention provides a method for folding protein and polypeptide S-cysteine derivatives in slightly basic buffers, at 37°C and in the presence of high concentrations of cysteine and low concentrations of chaotropic agents, such as guanidine salts, is provided.

An improved method has been developed for producing biologically active polypeptides and proteins consisting of the rapid solid-phase chemical synthesis of S-thio-t-butylated derivatives combined with a simple protocol of folding said protein and polypeptide derivatives in the presence of cysteine as a thiol catalyst.

The invention is a combination of :

1. rapid assembly of the protein and polypeptide S-thio-t-butylated chains on resin ;

2. cysteine-catalyzed thiol-disulfide exchange of the derivatives in slightly basic conditions leading to cysteinylated polypeptides and proteins that are the macromolecular oxidized forms (protein-S-S-cysteine ; polypeptide-S-S-cysteine) of the classical redox Cystine/Cysteine pair ;

3. a concentration of the oxidized macromolecular form which remains constantly low

throughout the folding process so that intermolecular disulfide exchange is minimized ;

4. lack of aggregation of misfolded intermediates due to preferential and rapid formation of forms with correct cysteine pairing (native structure).

Accordingly, the present invention provides a two step process for folding polypeptide and protein S-t-butyl derivatives prepared by chemical synthesis. In the first step, 10 mg of the S-t-butyl derivative are dissolved at room temperature in 1 ml of a buffer, pH 8.0, comprising 6 M of guanidinium chloride, 10 mM Tris and 0.1 M Na_2HPO_4. The resulting solution is maintained at room temperature for about 20 min.

In the second step, the solution is first diluted 10 fold with water to a pH = 7.2, (0.6 M guanidinium chloride, 1.0 mM Tris, 10 mM Na_2HPO_4 and final concentration of the polypeptide derivative 1 mg/ml) and then a strong molar excess of cysteine (about 100-fold over the concentration of the polypeptide or protein derivative) is added under stirring. The temperature is gradually increased to 37°C and maintained constant for about 24 h.

The folding method of the present invention results in a highly homogenous product and is applicable, with only minor modifications, for any polypeptide and protein which is produced by solid-phase chemical synthesis as the cysteine thio-t-butyl derivative. The advantages of this method of folding over the existing procedures employing precursor polythiol forms are :

a) cysteine residues of the chain are not alkylated during acidolytic cleavage of polypeptide-resin ;

b) both overoxidation of cysteine to sulphonic acid and oxidation leading to intermolecular disulfide bridge formation do not occur;

c) the risk of derivatization of the Trp indole ring by mercaptoethanol, which is necessary to eliminate

contaminating Hg ions deriving from Acm deblocking with $Hg(AcO)_2$, is avoided. The cysteine thiolate in the folding mixture of the present invention does not modify the Trp at all;

d) oxidation sensitive Met, Trp and Tyr residues are not modified during folding ;

e) costs of production of the final folded products are generally lower by adopting the methodology of the present invention as compared to those employing polythiol forms and redox buffers.

After folding, the target polypeptides and proteins can be purified by methods well known in the art including anion and cation exchange chromatography, hydrophobic interaction chromatography, reverse phase chromatography, affinity chromatography, Hydrophylic Interaction/Cation Exchange Chromatography (HILIC/CEC), Displacement Chromatography (DC) and Sample Displacement Chromatography (SDM). Most preferably, reverse phase high performance chromatography in elution as well as displacement mode are employed.

It will be apparent to those skilled in the art that, although target polypeptides and proteins are generally prepared in high yield using the process of the present invention, in some cases e.g. complex proteins with multiple disulfide bonds, a certain population of intermediate forms that have not completely evolved to the native structure (misfolded species) may remain in solution at equilibrium. Misfolded species can be easily separated from the correctly folded species by RP-HPLC and subjected again to the folding conditions of the present invention to increase the overall yield of the process.

The following examples are provided to illustrate the present invention and are not intended to limit the invention beyond the limitations set forth in the claims.

EXAMPLES

EXAMPLE 1

Synthesis and folding of Cys ^{10,11,34,50}(S-t-Bu)-hu-TARC (thymus and activation regulated chemokine).

The 71-amino acid residues long chemokine derivative was assembled on a 433 A Peptide Synthesizer (Perkin Elmer/ABI) using Fmoc/t-Bu chemistry and a polystyrene-based resin functionalized with the acid-labile hydroxymethylphenoxyacetic acid linker (Wang resin) on which Fmoc-Ser(t-Bu) was attached by DMAP (4-dimethylaminopyridine)-catalyzed esterification. The degree of substitution was 0.57 mmole/g . The synthesis was conducted on a 0.27 mmole scale using a five-fold excess of Fmoc-amino acids and DCI (N,N'-diisopropylcarbodiimide)/HOBt (1-hydroxybenzotriazole) activating reagents in DMF. The coupling time was about 60 min with spectrophotometric monitoring of Fmoc deprotection.

All the four cysteine thiols were protected with S-t-Butyl group.

A maximal protecting scheme was used for all other side-chains : Ser(t-Bu), Thr(t-Bu), Tyr(t-Bu), Asp(O-t-bu), Glu(O-t-Bu), Lys(Boc), Trp(Boc), Asn(Trt), Gln(Trt) and Arg(Pmc). After each coupling, capping with acetic anhydride and DIEA in DMF was carried out. The resulting polypeptide-resin was treated at room temperature with a freshly prepared mixture of TFA/water/TIS (triisopropylsilane)/phenol (78 :5 :12 :5, v/v/v/w, 10 ml/g resin) for 2.5- 3.0 h. The cleaved polypeptide derivative was precipitated by direct filtration of the cleavage mixture into cold methyl-t-butyl ether (MTBE) and the precipitate separated by centrifugation, washed twice with ether and dried in air.

The crude product was dissolved in diluted acetic acid, lyophilized, redissolved in 50% acetic acid and applied to a Sephadex G-50 column (70x25 cm) using 50% acetic acid as the mobile phase. The collected fractions were analyzed by MALDI-TOF mass spectrometry

and those containing the desired polypeptide derivative (MW 8,436.9 Da) were pooled and lyophilized after dilution with water.

The pooled fractions were again dissolved in 50% acetic acid and further purified by loading on a 250x10 mm semipreparative Vydac C_4 column. Samples were eluted at a flow rate of 3 ml/min with a linear gradient of 20-80% B in 60 min, where B was 0.1% TFA in acetonitrile and A 0.1% TFA in water. The detection was done at 280 nm and only the fractions containing the target polypeptide were pooled and lyophilized prior to folding.

Folding of the chemokine derivative purified by RP-HPLC was carried out by first dissolving 10 mg of product in 1 mg of 6M GnHCl, 0.1 M Na_2HPO_4 and 10 mM Tris at pH=8.0, room temperature. After 20 min, the solution was diluted by adding 10 ml of water to the final concentration of 0.6 M GnHCl, 10 mM Na_2HPO_4, 1 mM Tris, pH=7.2 and peptide concentration of 1mg/ml.

Folding was initiated by adding cysteine at a concentration of about 20 mM (about 100 fold molar excess with respect to the peptide concentration) and gradually increasing temperature to 37°C.

The folding reaction, occurring at the constant temperature of 37°C in air, was monitored by RP-HPLC analysis of 25 microliter aliquots of solution acid-quenched with acetic acid on a Waters 2690 Separation Module equipped with a Waters 996 Photodiode Array Detector, using a Vydac C_4 analytical column and a 20-60% acetonitrile gradient in 0.1% TFA/water in 40 min with a flow rate of 1.0 ml/min. 1 microliter of each HPLC peak (corresponding to the folding intermediates of the thiol-disulfide exchange reactions) was collected, mixed with 1 microliter of a saturated solution of sinapinic acid in 1 :2 acetonitrile/1% TFA in water, dried under vacuum into a well of the plate and analyzed by MALDI-TOF mass spectrometry, using a Voyager-DE spectrometer (Perseptive

Biosystem, Framingham, MA) equipped with a nitrogen laser.

78% of folded polypeptide formed after 24 h.

The peak whose MW corresponded to that of the folded product was further checked by reaction with N-ethylmaleimide (NEM) to detect the presence of free thiol groups (+125 Da for every SH).

The biological activity of hu-TARC obtained by the methodology of the present invention was performed according to the Imai method (T. Imai et al., J. Biol. Chem., 271, 21514, 1996).

Human T cell lines, Hut78, Hut 102, and Jurkat, as well as fresh monocytes, neutrophils and lymphocytes were assessed for their migration across a polycarbonate filter in response to TARC. No chemotactic response was elicited in monocytes or neutrophils both by TARC prepared by chemical synthesis and by recombinant TARC. In T cell lines Hut78 and Hut102, synthetic TARC as well as recombinant TARC induced migration with a typical bell-shaped curve with a maximum effect at 100 ng/ml.

EXAMPLE 2

Synthesis and folding of Cys $^{10, 34, 50}$(S-t-Bu)-hu-TARC and Cys $^{11, 34, 50}$(S-t-Bu)-hu-TARC

The synthesis, purification and folding of Cys $^{10, 34, 50}$(S-t-Bu) hu-TARC and Cys $^{11, 34, 50}$(S-t-Bu)- hu-TARC derivatives has been conducted in the same conditions adopted for Cys ^{10,11,34,50}(S-t-Bu) hu-TARC, the only difference being the Trt protection at Cys ^{10} and Cys ^{11}, respectively, which was removed concomitantly to cleavage of the polypeptide precursors from the resin. The yields of final folded chemokines was 80% and 79%, respectively.

EXAMPLE 3

Synthesis and folding of Cys $^{34, 50}$(S-Bu)-hu-TARC

The synthesis, purification and folding of Cys $^{34, 50}$(S-Bu) hu-TARC derivative was conducted in the same conditions of the derivatives of Example 1 and 2, except that both Cys 10 and 11 were protected by Trt which was removed during the final resin cleavage by TFA. The yield of folded product was about 75%.

EXAMPLE 4

Synthesis and folding of Cys^{10,11,26,34,50,68} (S-t-Bu)-hu-I-309

The synthesis of hu-I-309 containing 6 (S-t-Bu) protected cysteines was conducted on a 0.12 mmole scale in the same conditions as in example 1 using a Fmoc-Lys(Boc) Wang resin (degree of substitution of 0.61 mmol/g). The resulting polypeptide-resin was treated as described for example 1 and the G50 purified material was further purified by loading on a 250x10 mm Vydac C_{18} column.

Folding of the chemokine derivative purified by RP-HPLC was carried out by dissolving 65 mg of product in 60 ml of 0.6M GuHCl, 10 mM NaHPO4 and 1 mM Tris at pH=8.0 and adding cysteine at a concentration of 100 fold molar excess with respect to the peptide. Peptide solution was left at 37°C for 4 days. After acidulation with TFA the folded material was isolated by RP-HPLC using a 250x10 mm Vydac C_{18} column. Complete cysteine pairing was checked by mass spectrometry after reaction with N-ethylmaleimide (NEM). No MW increase was observed indicating absence of free thiol groups. The yield of final folded chemokine was almost 25%.

Chromatographic conditions of the figures of Example 4:

Analytical chromatography was performed using the following conditions:

Column: C18 250 x 4.6 mm (Vydac#238TP54)

Mobile phase: A = 100% H_2O 0.1%TFA

B = 100% CH_3CN 0.1%TFA

Gradient: B% composition is reported on the chromatogram

Detector: 214 nm

EXAMPLE 5

Synthesis and folding of Plasmodium vivax C-terminal fragment

The synthesis and purification of Plasmodium vivax circumsporozoite protein (PvCS) 303-372 containing 4 (S-t-Bu) protected cysteines was conducted in the same conditions as in example 1. Folding was performed by adding 27 mg of peptide in 2.7 ml of 6 M GuHCl in 0.1 M Tris buffer, pH 8.5. Mix for 10 min. Then add 13.5 ml of 1 mM EDTA, 0.2 M NaCl buffered at pH 8.8 in 0.2 M Tris buffer.Add 10.8 ml of 35 mM cysteine in 1 mM EDTA, 0.2 M NaCl buffered at pH 8.8 in 0.2 M Tris buffer. Bring the reaction mixture at 37° C Follow the folding reaction on reverse phase HPLC to completion (3-6 h). Stop the reaction by cooling for 5 minutes at 4° C followed by addition of 10% TFA at 4° C to reach a final concentration of 1% TFA (3 ml of 10% TFA).

The product is then purified by reverse phase HPLC. The yield of the final oxidized product is 70-80%.

Chromatographic conditions of the figures of Example 5:

Analytical chromatography was performed using the following conditions:

Column: C4 250 x 4.6 mm (Vydac#214TP54)

Mobile phase: A = 100% H_2O 0.1%TFA

B = 100% CH_3CN 0.1%TFA

Gradient: B% composition is reported on the chromatogram

Detector: 214 nm

CLAIMS

1. A process for the production of folded-biologically-active forms of polypeptides and proteins, in which derivatized cysteines are progressively changed to cystines while forming disulfide bridges by adding an excess of a reducing agent in defined buffers, temperature and chaotropic salts.

2. The process in claim 1 wherein the polypeptides and proteins are prepared by chemical synthesis.

3. The process in claim 1 wherein the derivatized cysteines correspond to S-t-butyl-thio-cysteine derivative.

4. The process in claim 1 wherein the reducing agent is cysteine and is added in excess to the S-t-butyl-thio-cysteine derivative.

5. The process in claim 1 wherein the defined buffer is a slightly basic solution.

6. The process in claim 1 wherein the defined temperature is between 25 and 37°C.

7. The process in claim 1 wherein chaotropic salts is guanidinium chloride or urea at 0.5-1.0 M concentration.

8. The process according to claim 1 in which the biologically active forms of polypeptides and proteins are produced by the steps of:

a) assembling the S- t-butyl-thio cysteine chains on insoluble polymeric supports by stepwise chain elongation;

b) cleaving the polypeptide-resin derivatives by acidolysis in the presence of scavengers and purifying the products by High Performance Liquid Chromatography;

c) folding the purified polypeptide derivatives in buffers containing a low concentration of a chaotropic salt, at slightly basic pH and 37°C, upon addition of a large molar excess of cysteine;

d) recovering the folded native polypeptides and proteins by High Performance Liquid Chromatography.

9. The process according to claim 8, wherein in step a) said polymeric supports are polyamide or polystyrene-based resins functionalized with the acid labile hydroxymethylphenoxyacetic acid linker and the chain is built up utilizing the mild 9-fluorenyl-methyloxycarbonyl/t-butyloxycarbonyl-based scheme for the amino acids.

10. The process according to claim 8, wherein in step b) the acidolytic cleavage is made with a mixture of TFA, water, triisopropylsilane and phenol as scavengers (TFA/H_2O and TFA/ triisopropylsilane volume ratios 90/2 and 90/8, respectively ; phenol, 5% w/v).

11. The process according to claim 8, wherein the chaotropic salt is guanidinium chloride.

12. The process according to claim 8, wherein the buffer is composed of 0.6 M guanidinium chloride, 10 mM Na_2HPO_4 and 1mM Tris, pH=7.2.

13. The process according to claim 8, wherein the S-thio-t-butyl derivative is first dissolved at a concentration of about 10 mg/ml in 0.1 M Na_2HPO_4, 10 mM Tris, 6M guanidinium chloride buffer, at pH=8.0; the solution, maintained at room temperature for about 25 min, is then diluted with 10 volumes of water to bring pH to 7.2 and the concentration of the peptide derivative to 1mg/ml.

14. The process according to claim 8, wherein in step c) the folding is initiated by the addition of cysteine at a concentration of about 20 mM, at the molar ratio of cysteine and polypeptide derivative of 100/1, while the temperature is gradually increased to 37°C.

15. The process according to claim 8, wherein in step c) the temperature is maintained at 37°C for about 24 h.

16. The process according to claim 8, wherein in step d) the folded polypeptides and proteins are preferably recovered by Reverse-Phase High Performance

Liquid Chromatography, operating both in elution and Sample Displacement Mode.

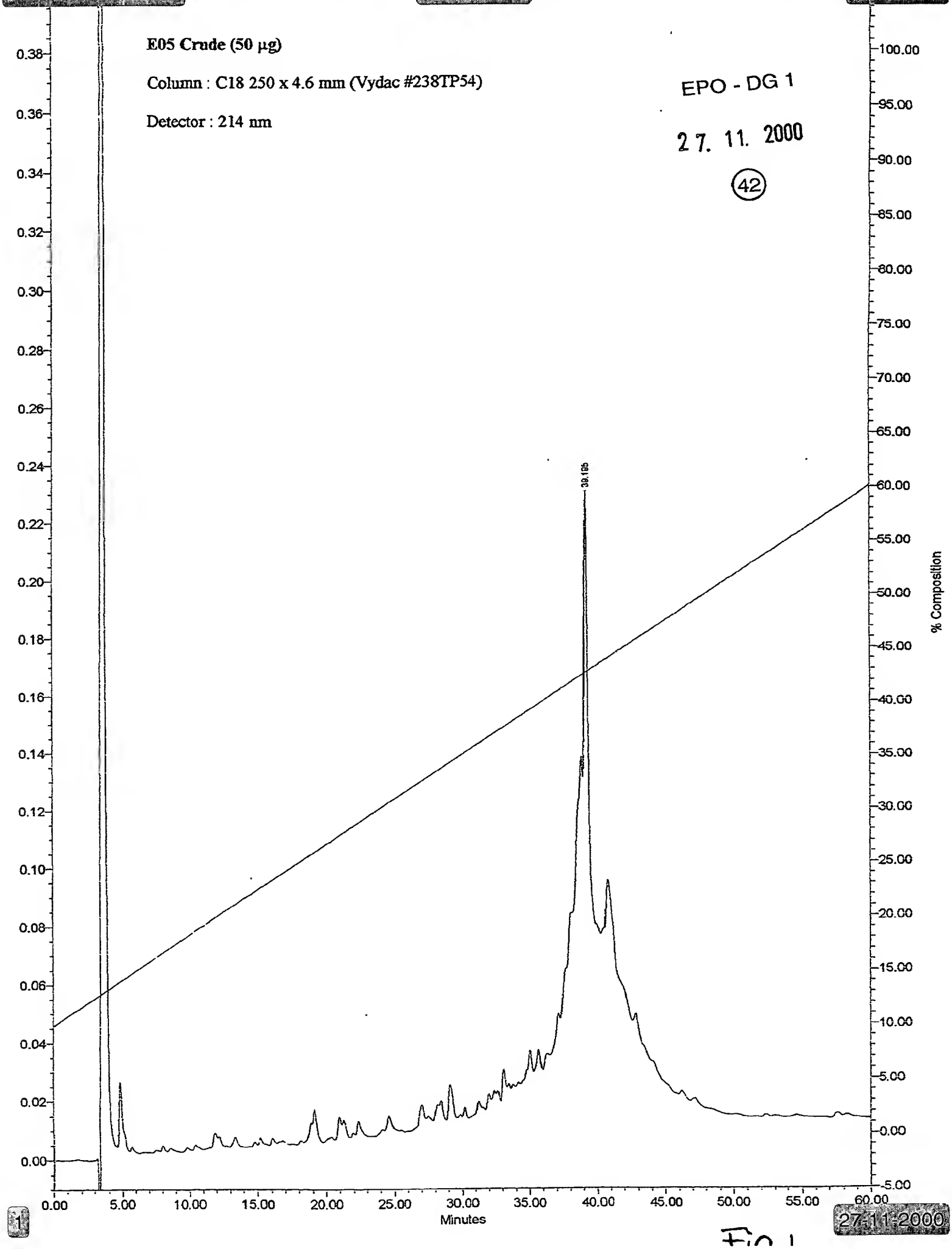

Fig 1

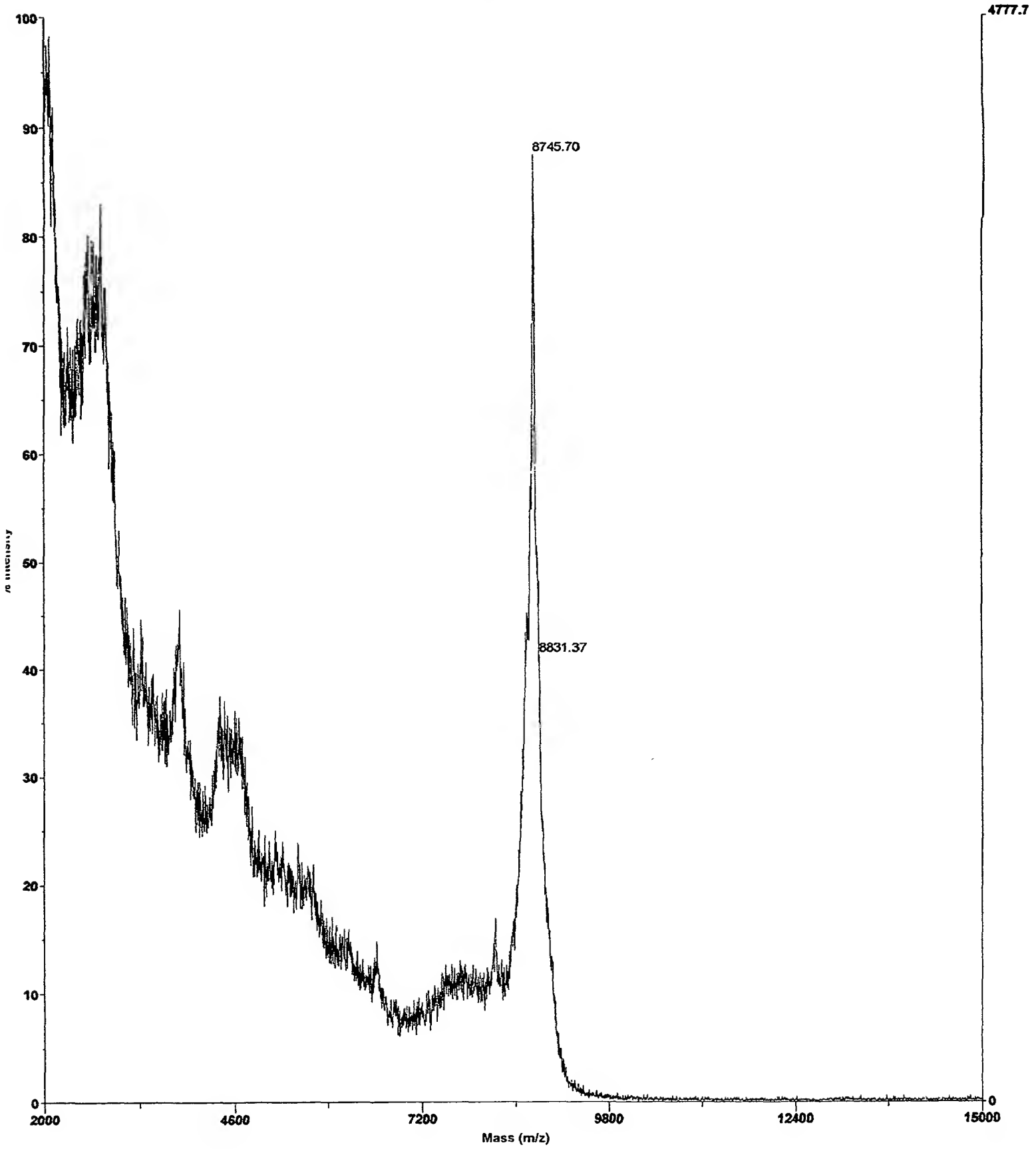

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Fig.2

27-11-2000

E05 Folded Peak II

Column : C18 250 x 4.6 mm (Vydac #238TP54)

Detector : 214 nm

	Retention Time	Area	% Area
1	24.595	14664	0.11
2	25.462	12048	0.09
3	26.894	237213	1.84
4	27.798	12579793	97.83
5	29.801	15144	0.12

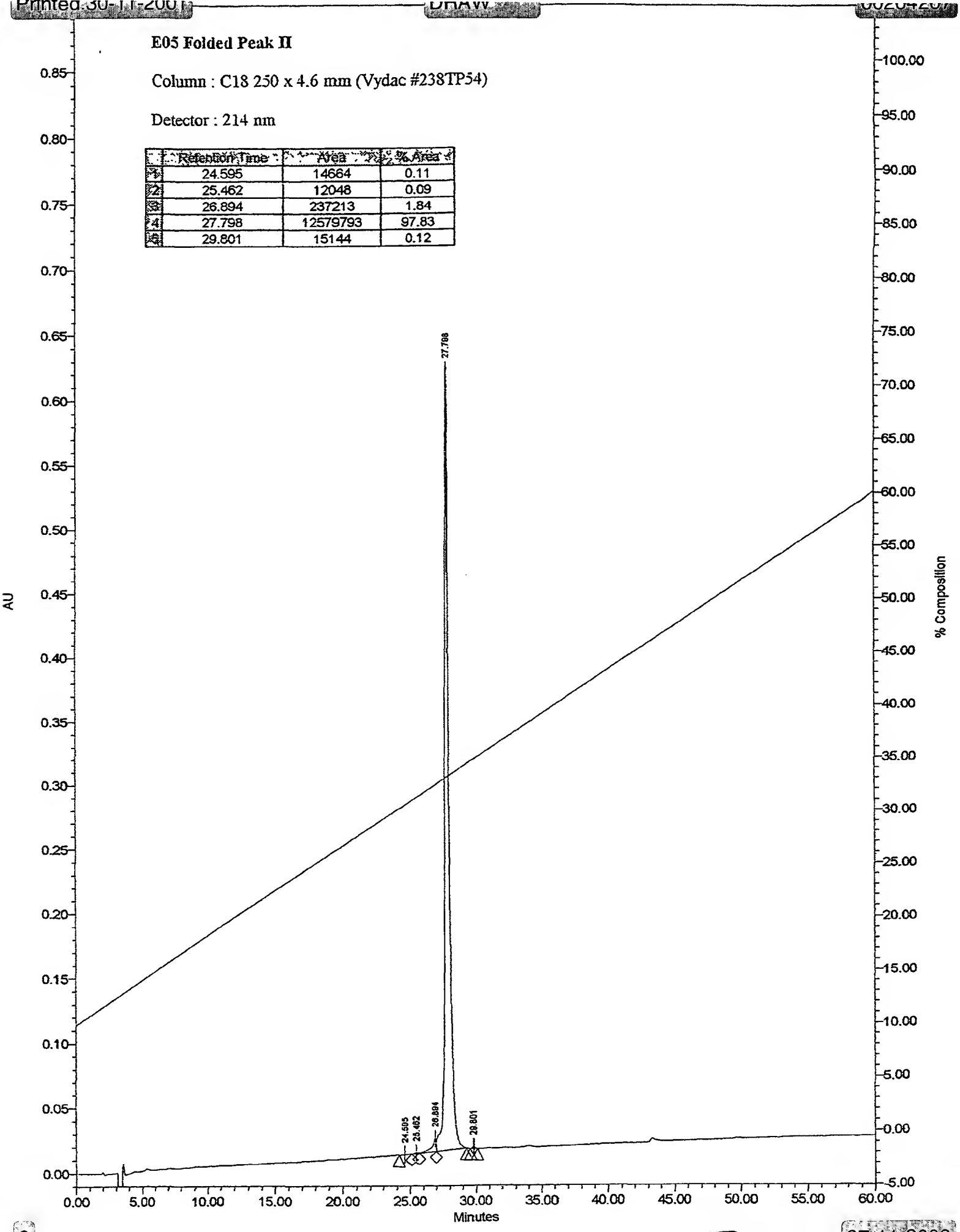

Fig.3

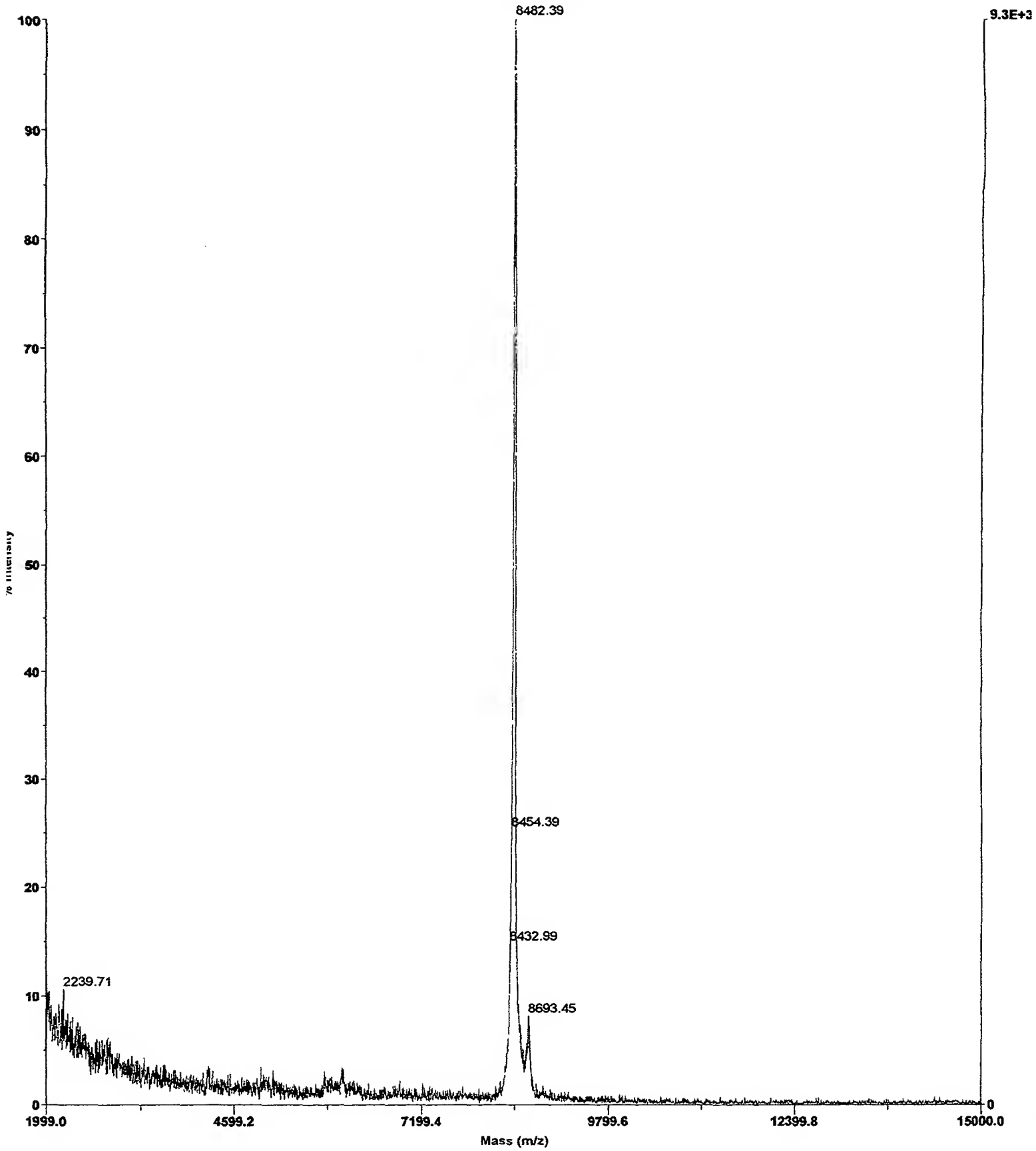

Fig.4

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27-11-2000

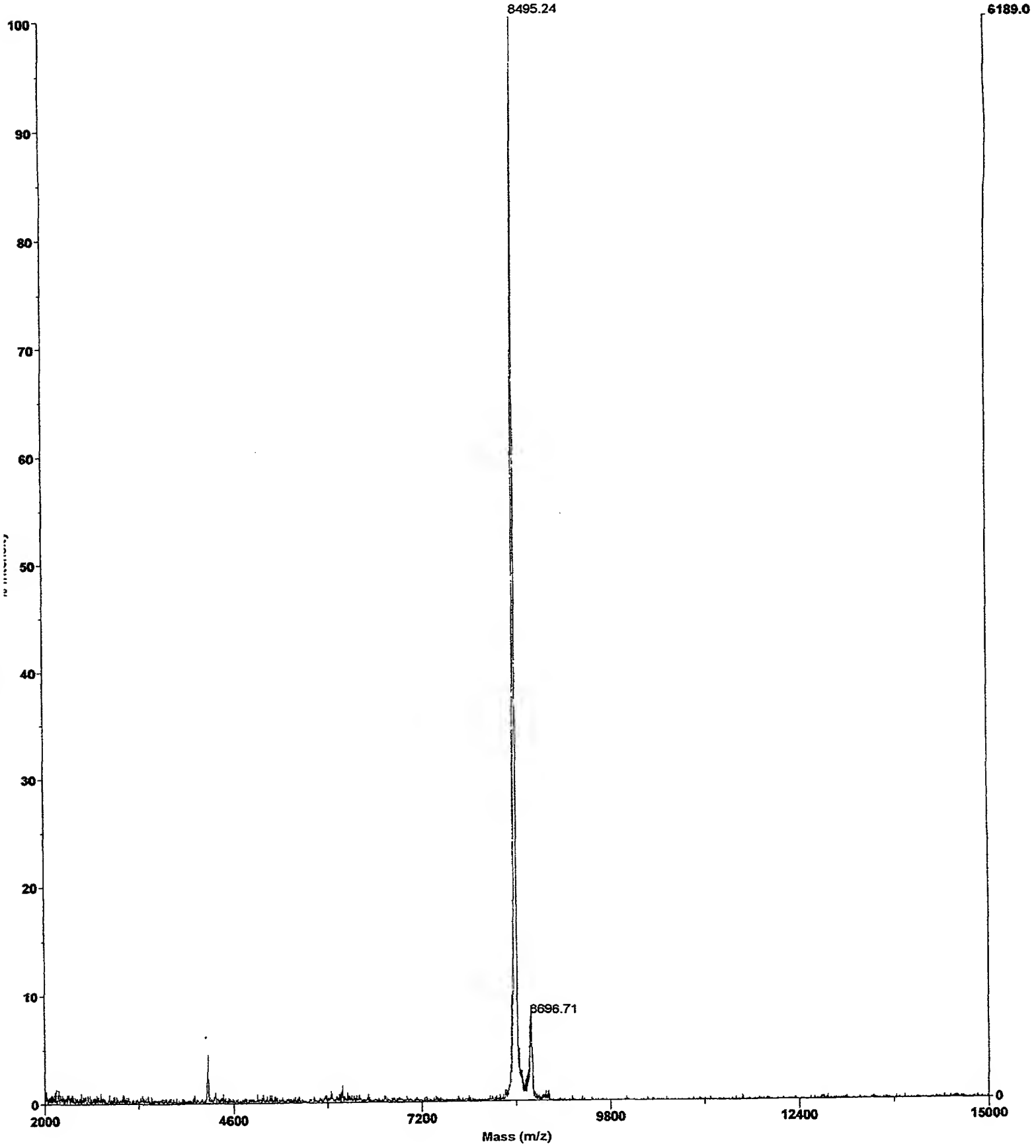

Fig.5

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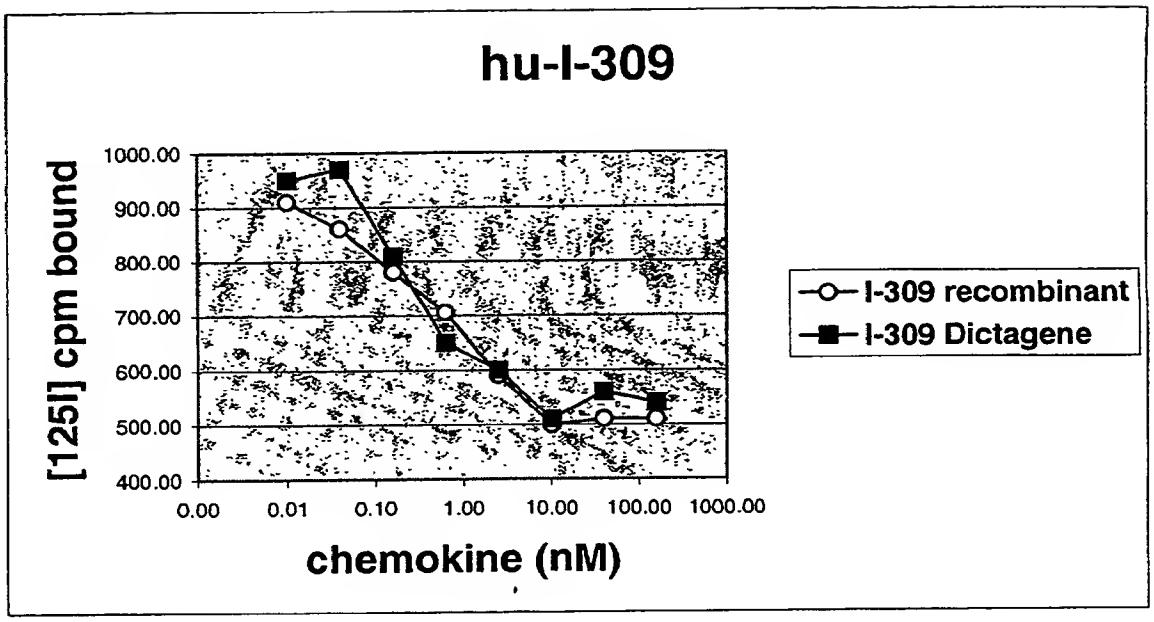
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400.00
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0.01
0.10
1.00
10.00
100.00
1000.00
chemokine (nM)
I-309 recombinant
I-309 Dictagene

Fig.6

==========

Area Percent Report

==========

Sorted By : Signal
Multiplier : 1.0000
Dilution : 1.0000

Signal 1: VWD1 A, Wavelength=214 nm

Peak #	RetTime [min]	Type	Width [min]	Area mAU *s	Height [mAU]	Area %
1	2.677	PV	1.2955	2.92536e5	2695.09839	92.5897
2	6.306	VB	1.3318	5152.31250	48.30238	1.6307
3	13.570	BV	0.2106	1.69669e4	1073.30298	5.3701
4	15.000	VV	0.4958	1293.41760	35.67244	0.4094

Totals : 3.15949e5 3852.37619

Results obtained with enhanced integrator!

==========

Summed Peaks Report

==========

Signal 1: VWD1 A, Wavelength=214 nm

==========

Final Summed Peaks Report

==========

Signal 1: VWD1 A, Wavelength=214 nm

*** End of Report ***

Fig. 7A

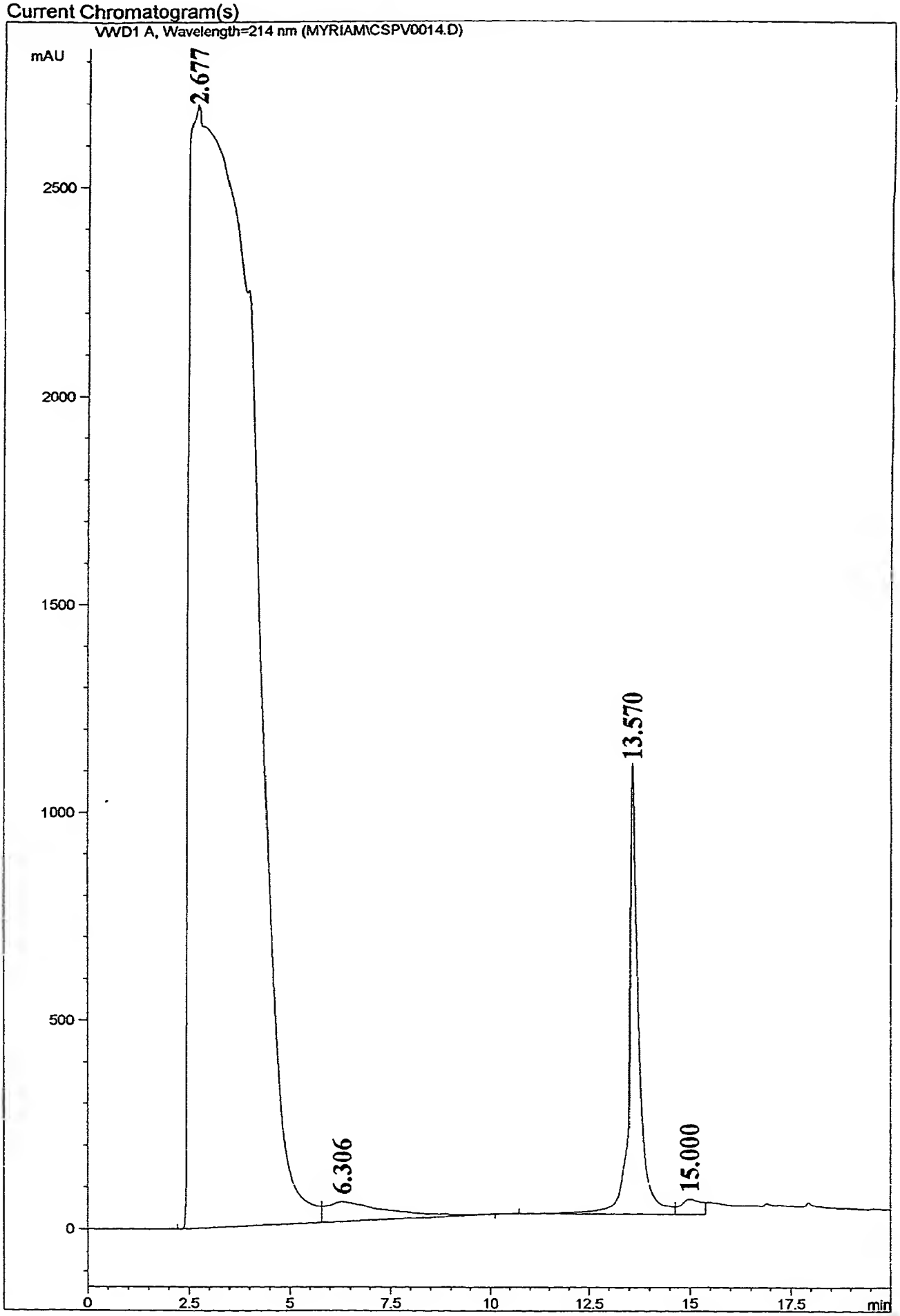


Fig. 2B

27-11-2000

ent Chromatogram(s)

DRAW

PUCS BON (= FINAL)

Current Chromatogram(s)

VWD1 A, Wavelength=214 nm (MYRIAM\CSPV0014.D)

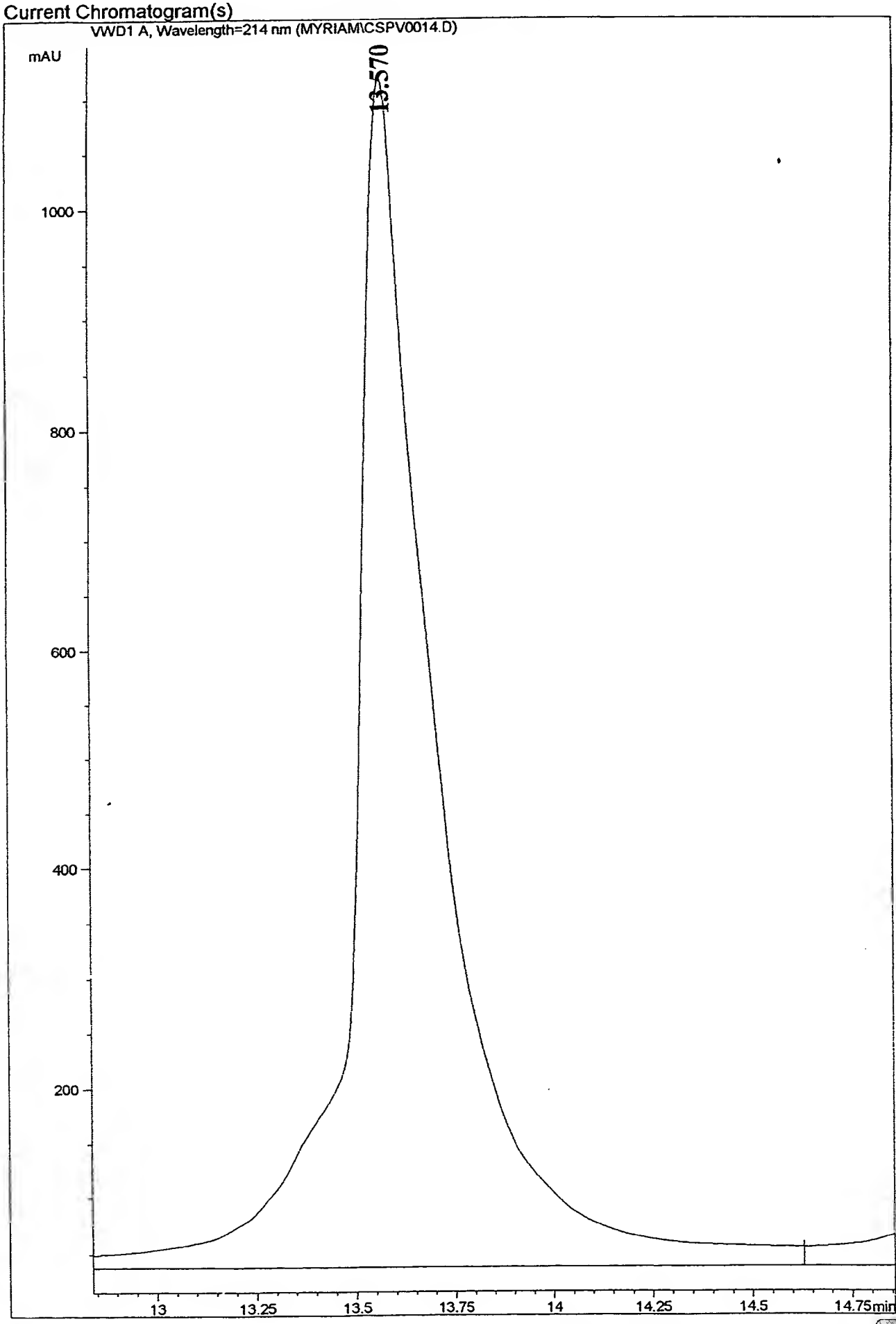
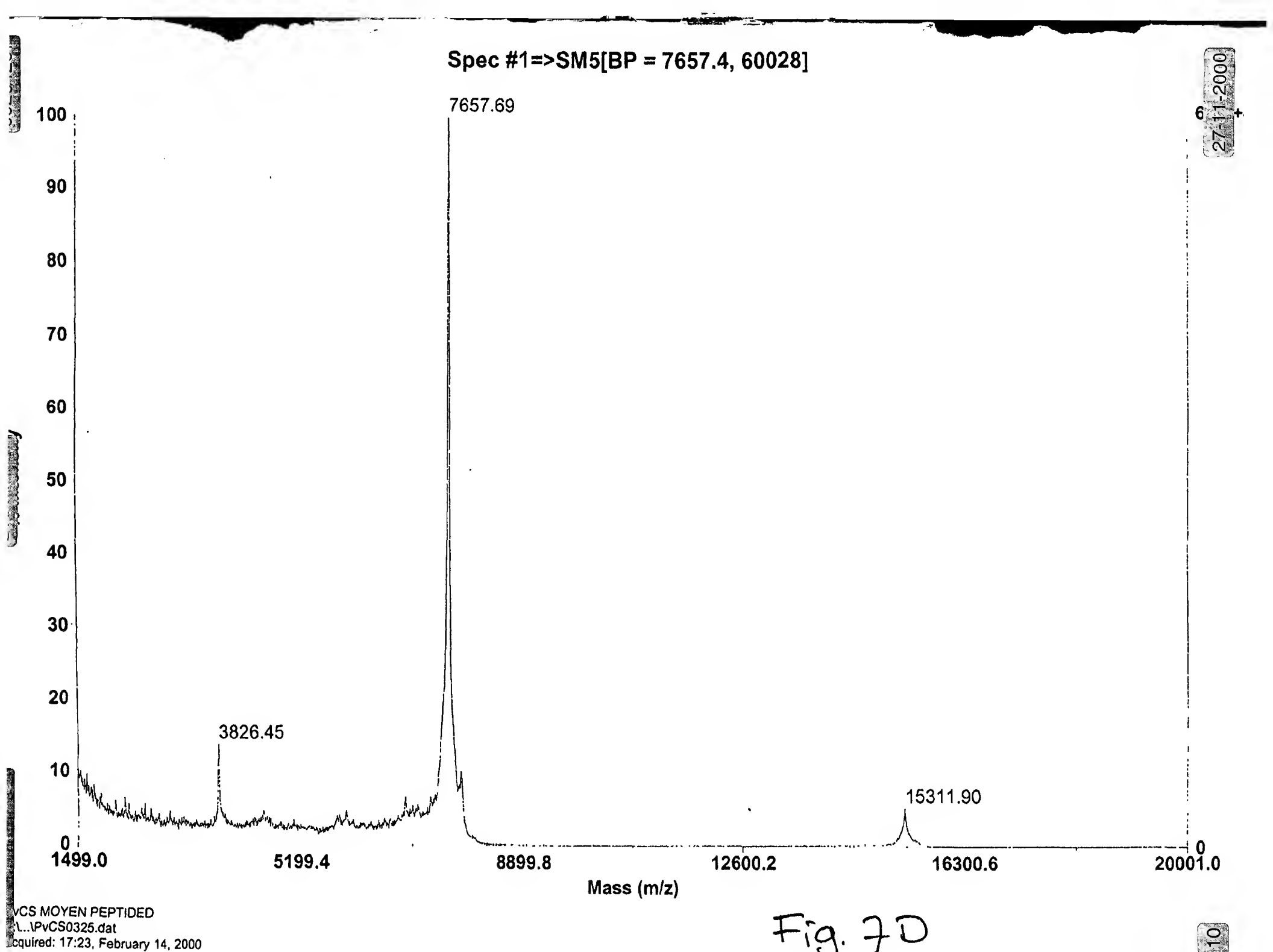


Fig. 7C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12600.2
16300.6
20001.0
Mass (m/z)
27-11-2000
6
+
VCS MOYEN PEPTIDED
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cquired: 17:23, February 14, 2000
10

Fig. 7D